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Mohammadi, M.,-Dionne, C., Li, W., Li, N., Spivak, T., Honegger, A., Jaye, M. and Schlessinger, J. (1992). Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis. Nature 358, 681-684.

Peters, K., Marie, J., Wilson, E., Ives, H., Escobedo, J., Del Rosario, M., Mirda, D. and Williams, L. (1992). Point mutation of an FGF receptor abolishes phosphatidylinositol turnover and Ca.sup.2+ flux but not mitogenesis. Nature 358, 678-681.

Thanks a lot...

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phorylation secondary to defective activation of an intracellular serine/threonine kinase. Phosphorylation of tyrosine kinase receptors by serine/threonine kinases is thought to have a negative regulatory effect and might also explain an increase in the kinase activity of the mutant receptor[18,19].

Given the proposed importance of PtdIns turnover and Ca^{2+} mobilization in growth factor-stimulated processes and the profound effect FGF treatment of cells has on these pathways, we were surprised to find that FGF stimulated mitogenesis in cells expressing the Y/F766 mutant as well or better than in cells expressing the wild-type receptor. DNA synthesis increased significantly in all cell lines tested (Fig. 4*c*) and cell numbers increased in proportion to DNA synthesis (data not shown).

PLCγ SH2 domains can bind to Tyr 992 in the C-terminal tail of the epidermal growth factor (EGF) receptor, and a C-terminal tail of a truncated EGF receptor which is also mutated at Tyr 992 still phosphorylates PLCγ but does not activate PtdIns turnover[20,21]. Also, truncated EGF receptors lacking multiple potential autophosphorylation sites, including Tyr 992, do not associate with or phosphorylate PLCγ, are defective in EGF-mediated receptor downregulation, and can transform cells[22–25]. The colony-stimulating factor (CSF-1) receptor can mediate mitogenesis in fibroblasts without phosphorylating or activating PLCγ (ref. 26). Overexpression of PLCγ gives increased PtdIns turnover in response to FGF or PDGF but does not enhance mitogenesis in response to either growth factor, suggesting that PtdIns turnover is not limiting in the mitogenic response to FGF or PDGF[27,28]. Our data demonstrate that increased PtdIns turnover and intracellular calcium mobilization are not required for mitogenesis in response to FGF in L6 myoblasts. Thus other signalling pathways must be involved in mitogenesis by the FGF receptor.

If not required for mitogenesis in response to FGF, what other cellular responses might be triggered by PtdIns turnover? Recent studies suggest that the activation of PLCγ might mediate chemotaxis or cell-shape changes[29]. Others indicate that PtdIns turnover in response to FGF might mediate cellular differentiation during early embryonic pattern formation[30]. If the Y/F766 mutant does indeed have a selective signalling defect, it should be useful in establishing the role of FGF-mediated PtdIns turnover in chemotaxis, cell differentiation and other non-mitogenic cellular responses. □

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Point mutation in FGF receptor eliminates phosphatidylinositol hydrolysis without affecting mitogenesis

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STIMULATION of growth factor receptors with tyrosine kinase activity is followed by rapid receptor dimerization, tyrosine autophosphorylation and phosphorylation of signalling molecules such as phospholipase C_γ(PLCγ) and the *ras* GTPase-activating protein[1,2]. PLCγ and GTPase-activating protein bind to specific tyrosine-phosphorylated regions in growth factor receptors[3–9] through their *src*-homologous SH2 domains[7,8,10,11]. Growth factor-induced tyrosine phosphorylation of PLCγ is essential for stimulation of phosphatidylinositol hydrolysis *in vitro*[12] and *in vivo*[13]. We have shown that a short phosphorylated peptide containing tyrosine at position 766 from a conserved region[14–18] of the fibroblast growth factor (FGF) receptor is a binding site for the SH2 domain of PLCγ (ref. 8). Here we show that an FGF receptor point mutant in which Tyr 766 is replaced by a phenylalanine residue (Y766F) is unable to associate with and tyrosine-phosphorylate PLCγ or to stimulate hydrolysis of phosphatidylinositol. Nevertheless, the Y766F FGF receptor mutant can be autophosphorylated, and can phosphorylate several cellular proteins and stimulate DNA synthesis. Our data show that phosphorylation of the conserved Tyr 766 of the FGF receptor is essential for phosphorylation of PLCγ and for hydrolysis of phosphatidylinositol, but that elimination of this hydrolysis does not affect FGF-induced mitogenesis.

To study the role of Tyr 766 in FGF receptor signalling, we generated transfected cell lines expressing either wild-type or three different FGF receptor mutants. The mutant FGF receptors included a point mutant in which Tyr 766 was replaced by a phenylalanine residue (Y766F), a control mutant in which an adjacent non-phosphorylated Tyr 776 was replaced by a phenylalanine residue (Y776F), and a double mutant in which both Tyr 766 and Tyr 776 were substituted by phenylalanine residues (Y766/776F). Wild-type or mutant FGF receptors were expressed in transfected L6 myoblasts lacking endogenous FGF receptors. Several cell lines expressing each mutant receptor were generated and characterized. These cell lines were treated with acidic FGF, lysed, immunoprecipitated with anti-FGF receptor antibodies and, after SDS–PAGE, immunoblotted with either anti-FGF receptor or anti-phosphotyrosine antibodies (Fig. 1). This experiment shows that, in response to acidic FGF, both wild-type and mutant FGF receptors undergo tyrosine autophosphorylation. We next compared typtic phosphopeptide maps of wild-type and mutant FGF receptors. Figure 2 shows that the tryptic digest of wild-type FGF receptor contains three phosphopeptides and that phosphopeptide P1 (Fig. 2*a*), which contain Tyr 766 (ref. 8), is missing from the trypic digests of FGF receptor mutants Y766F and Y766/776F.

We next examined the capacity of mutant FGF receptors to associate with and tyrosine-phosphorylate PLCγ . Figure 3 shows that only wild-type FGF receptor and the FGF receptor Y776F mutant could associate with (Fig. 3*a*, *b*) and mediate tyrosine phosphorylation (Fig. 3*c*, *d*) of PLCγ in living cells. In contrast, no tyrosine phosphorylation of PLCγ was detected in cells expressing either Y766F or Y766/776F mutants. As growth factor-induced tyrosine phosphorylation of PLCγ is

essential for its activation[12,13], it was expected that FGF receptor mutant Y766F would not be able to stimulate phosphatidylinositol (PtdIns) hydrolysis. Addition of acidic FGF to L6 myoblasts expressing either wild-type or control Y776F FGF receptor mutant led to a large increase in PtdIns hydrolysis (Fig. 4*a*). As anticipated, acidic FGF was unable to stimulate PtdIns hydrolysis in cells expressing either Y766F or Y766/776F FGF receptor mutants. Hence, elimination of Tyr 766 prevents FGF-induced tyrosine phosphorylation of PLCγ and PtdIns hydrolysis.

Growth factors such as FGF, epidermal growth factor (EGF) or platelet-derived growth factor (PDGF) stimulate association with and tyrosine phosphorylation of PLCγ and subsequent enhancement of PtdIns hydrolysis[3-9,12,13,19-21]. The role of

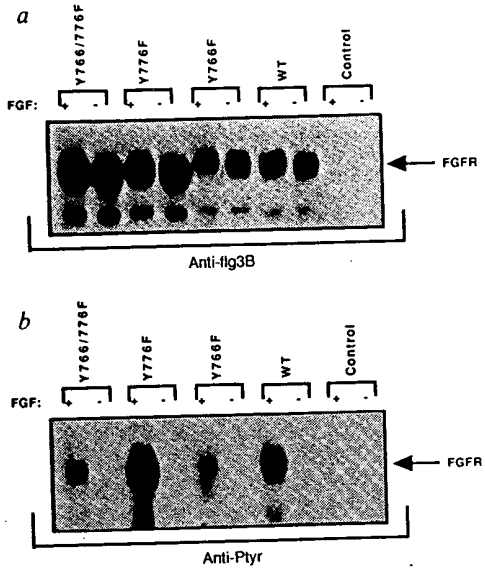

FIG. 1 Acidic FGF-induced tyrosine autophosphorylation of wild-type (WT) and mutant FGF receptors in living cells. Transfected L6 myoblast cell lines expressing either wild-type or FGF receptor (FGFR) mutants were treated in the presence (+) or absence (−) of acidic FGF, lysed and immunoprecipitated with anti-FGF receptor (anti-flg1A) antibodies followed by SDS–PAGE and immunoblotting with *a*, anti-FGF receptor (anti-flg3B) or *b*, antiphosphotyrosine antibodies (anti-Ptyr).

METHODS. Site-directed mutagenesis was performed according to the manufacturer's protocol (Amersham). A cDNA encoding the full-length human FGF receptor[18] was subcloned in m13MP19 replicative form using *Bam*HI–*Hin*dIII. Point mutations, which changed Tyr 766 and Tyr 776 either individually or in combination to phenylalanine residue(s) were introduced using oligonucleotides 5′-CCTCCAACCAGGAGTTCCTGGACCTGTCCATG-3′ (for Y766F mutant), 5′-TGCCCCTGGACCAGTTCTCCCCCAGCTTTC-3′ (for Y776F mutant), and 5′-CAGGAGTTCCTGGACCTGTCCATGCCCCTGGACCAGTTCTCCCCC-3′ (for Y766/776F mutant). The cDNA encoding either wild-type or mutant FGF receptors were subcloned into the eukaryotic expression vector pMJ30, under the control of the adenovirus major late promoter and cytomegalovirus enhancer[33]. L6 rat myoblasts without endogenous FGF receptors were a subclone selected for high fusion. These cells were transfected with 0.5 μg pSV2neo and 20 μg wild-type or mutant FGF receptor expression vectors using calcium phosphate precipitation[34]. Clones were isolated after 2–3 weeks selection in G418 (Gibco) and screened for expression of FGF receptor by immunoprecipitation with anti-flg1A and immunoblotting with anti-flg3B antibodies. Binding experiments with ^{125}I-labelled FGF followed by Scatchard analysis showed that the L6 cells expressing wild-type FGF receptors have $\sim 4.5\times10^5$ receptors per cell, Y766F cells have 4.2×10^5, Y776F cells 5.6×10^5, and Y766/776F cells $\sim 5.8\times10^5$. The dissociation constant for the binding of FGF to all cell lines was $\sim 0.2\times10^{-9}$ M. Transfected L6 cells were stimulated with acidic FGF (100 ng ml^{-1}) for 5 min at 37 °C. Cells were washed twice with PBS (Gibco) and scraped into 0.5 ml lysis buffer containing phosphatase inhibitors as described[5]. Lysates were immunoprecipitated with anti-flg1A antibodies immobilized on protein A–Sepharose beads. Immunocomplexes were washed three times with HNTG (20 mM HEPES, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol), 3× Laemmli buffer was added and the samples boiled for 5 min. After analysis by SDS–PAGE, proteins were transferred electrophoretically to nitrocellulose membranes and immunoblotted with either anti-flg3B (1:100) or with rabbit polyclonal antiphosphotyrosine antibodies (1:100). The remaining tyrosine-phosphorylated sites on the Y766F mutant were poorly recognized by antiphosphotyrosine antibodies. Blots were treated with ^{125}I-labelled protein A and analysed by autoradiography. Rabbit anti-flg3B antiserum was generated against a synthetic peptide from the kinase-insert region (residues 580–586) of human FGF receptor. Anti-flg1A antiserum was generated against a synthetic peptide derived from the C-terminal tail of human FGF receptor (residues 808–822).

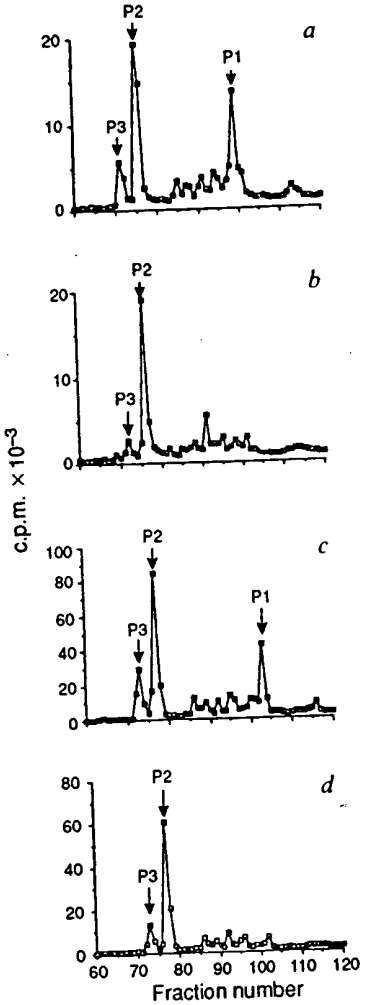

FIG. 2 Comparison of tryptic phosphopeptide maps of wild-type and mutant FGF receptors. Analysis by reverse-phase HPLC of tryptic phosphopeptides of wild-type (*a*) and FGF receptor mutants Y766F (*b*), Y776F (*c*) and Y766/776F (*d*). Fractions 60–120 contain the previously identified tryptic phosphopeptide P1 (arrow Y766)[8] and two uncharacterized phosphopeptides, P2 and P3.

METHODS. L6 myoblasts expressing wild-type, Y766F, Y776F or Y766/776F receptor mutants were stimulated with or without acidic FGF (100 ng ml^{-1}) for 5 min at 37 °C, lysed, and the soluble lysates immunoprecipitated with anti-flg1A antibodies. Kinase activity was assayed in 50 ml HNTG containing 5 mM Mn^{2+}, 1 μM unlabelled ATP and 5 μl [γ-^{32}P]ATP (6,000 Ci mmol^{-1}) for 10 min at 25 °C. Reaction products were analysed by SDS–PAGE (5–15%) followed by autoradiography. The FGF receptor-specific bands were excised from the SDS gel and digested with trypsin as described[8]. The eluted tryptic peptides were filtered (Millipore) and incubated with monoclonal antiphosphotyrosine antibodies (Oncogene Science) immobilized on beads for 2 h at 4 °C. The phosphotyrosine-containing peptides were eluted with 1 ml 50 mM phenylphosphate in 20 mM HEPES, pH 7.5, and separated on an Aquapore C18 (4.6 by 250 mm) reverse-phase HPLC column as described[8]. Note that the FGF receptor contains two uncharacterized tyrosine-phosphorylation sites (P2 and P3) in addition to Tyr 766 in the P1 phosphopeptide. All three phosphopeptides were also detected on *in vivo* autophosphorylation of FGF receptor in stably or transiently transfected cell lines (data not shown).

growth factor-induced PtdIns hydrolysis as part of signal transduction pathways required for mitogenesis is not clear, however[22-26]. The fact that FGF did not stimulate PtdIns hydrolysis in untransfected L6 or in L6 expressing the Y766F mutant offered a unique opportunity to dissect the role of PtdIns hydrolysis in FGF-induced motogenesis. Figure 4*b* shows that all transfected L6 myoblasts expressing either wild-type or mutant FGF receptors were mitogenically responsive to acidic FGF with a similar dose dependence. The inability of mutant FGF receptors to stimulate PtdIns hydrolysis did not influence their capacity to mediate a mitogenic signal. We therefore conclude that PtdIns hydrolysis is not required for FGF-induced mitogenesis.

Very little is known about FGF receptor signalling pathways

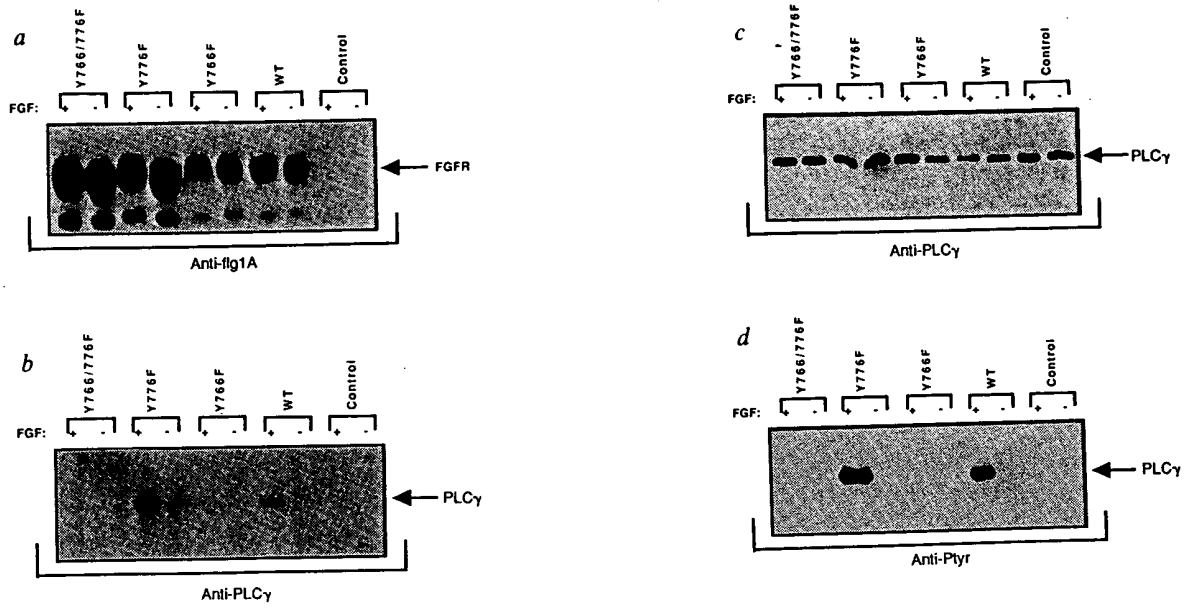

FIG. 3 Point mutation at Tyr 766 of the FGF receptor abolishes association with and tyrosine phosphorylation of PLCγ. L6 myoblasts expressing either wild-type or the three FGF receptor mutants were stimulated with acidic FGF, lysed and immunoprecipitated with anti-FGF receptor antibodies. Samples were analysed by SDS–PAGE and immunoblotted with either anti-FGF-receptor (*a*) of anti-PLCγ antibodies (*b*). Transfected L6 myoblasts expressing either wild-type or various FGF receptor mutants were incubated in the presence (+) or (absence (−) of FGF. Lysed cells were immunoprecipitated with anti-PLCγ antibodies followed by SDS–PAGE and immunoblotting with either anti PLCγ (*c*) or antiphosphotyrosine antibodies (*d*).

METHODS. Serum-starved L6 myoblasts or L6 expressing wild-type, Y766F, Y776F or Y766/776F receptor mutants were stimulated with (+) or without (−) FGF (100 ng ml^{-1}) for 5 min at 37 °C, lysed and the soluble lysates were immunoprecipitated with anti-flg3B antibodies. After SDS–PAGE, samples were immunoblotted with either anti-PLCγ or anti-flg1A antibodies. In parallel, lysates from FGF-treated and unstimulated cells were immunoprecipitated with anti-PLCγ antibodies, analysed by SDS–PAGE and immunoblotted with either anti-PLCγ or antiphosphotyrosine antibodies. Rabbit anti-PLCγ antiserum was raised against a synthetic C-terminal peptide of rat PLCγ (residues 1,256–1,274).

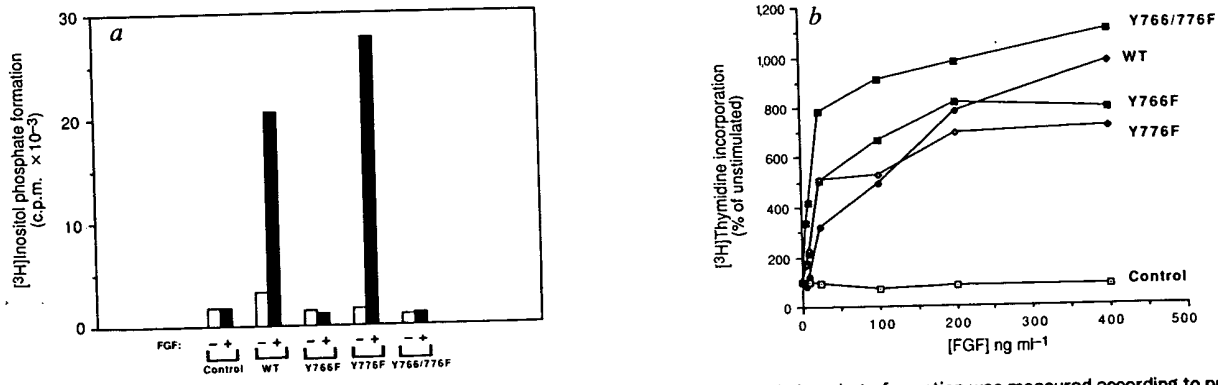

FIG. 4 A point mutation at Tyr 766 of the FGF receptor abolishes FGF-mediated PtdIns hydrolysis without affecting DNA synthesis. *a*, Inositol phosphate accumulation in unstimulated (open bars) or FGF-stimulated cells (black bars). Data represent the average of triplicate experiments for each cell type. *b*, Serum-starved cells were stimulated with increasing concentrations of acidic FGF. Average values of percentage increase in thymidine incorporation of three different experiments are presented.

METHODS. Phosphatidylinositol hydrolysis: L6 myoblasts expressing either wild-type or FGF receptor mutants were labelled with [^{3}H]myoinositol (2 μCi ml^{-1}) in DMEM containing 0.5% FBS for 24 h and incubated in DMEM containing 20 mM LiCl for 20 min before addition of acidic FGF (100 ng ml^{-1}) for an additional 30 min at 37 °C. Cells were extracted with 5% perchloric acid and inositol phosphate formation was measured according to published procedures[22]. Thymidine incorporation: L6 cells were seeded in 96-well plates (2×10^4 cells per well) and 24 h later the medium was changed to DMEM containing 0.1% FBS. After 48 h serum starvation, the medium was replaced by medium containing FGF or, as a control, 10% FCS. After stimulation for 24 h, cells were incubated with [^{3}H]thymidine (0.5 μCi ml^{-1}) for 16 h at 37 °C. Cells were washed with PBS, trypsinized and collected using a PHD Cell Harvester (Cambridge Technologies) and the amount of incorporated [^{3}H]thymidine was quantitated by liquid scintillation counting (LKB). Acidic FGF was also able to stimulate the proliferation of the various transfected L6 cell lines. Similar results were obtained with transfected 3T3 cells expressing endogenous FGF receptors (data not shown).

that may be required for FGF-induced mitogenesis. Signalling molecules such as PtdIns 3-OH kinase or *ras* GTPase-activating protein (GAP) that become associated with many activiated growth factor receptors do not seem to interact with the activated FGF receptor (ref. 27, and our unpublished results). Moreover, it is not yet clear whether downstream serine/threonine kinases such as Raf or Map2 kinase are activated on stimulation of FGF receptors. We have shown here that the intrinsic protein tyrosine kinase activity of the Y766F mutant is maintained and that this mutant is able to undergo autophosphorylation on at least two additional uncharacterized tyrosine residues (Fig. 2). It is therefore likely that the interaction between FGF receptor and substrates crucial for FGF-induced mitogenesis is not dependent on autophosphorylation of Tyr 766. Indeed, several proteins are tyrosine-phosphorylated in Y766F cells in response to FGF stimulation (data not shown).

Our main conclusions are that elimination of Tyr 766 of the FGF receptor abolishes PLCγ association with the receptor, PLCγ tyrosine phosphorylation and PtdIns hydrolysis and that PtdIns hydrolysis is not required for FGF-induced mitogenesis. This is also consistent with studies showing that colony-stimulating factor 1, insulin-like growth factor 1 and insulin are all able to stimulate DNA synthesis although these growth factors do not stimulate PLCγ phosphorylation and PtdIns hydrolysis[1,28]. In general then, PtdIns hydrolysis may not be required for mitogenesis triggered by activation of receptors with tyrosine kinase activity. Yet growth factors such as FGF, PDGF and EGF are clearly able to stimulate PtdIns hydrolysis. Hence, FGF-induced PtdIns hydrolysis could be crucial for the regulation of other non-mitogenic responses mediated by FGF. For example, FGF-induced PtdIns hydrolysis could be involved in the regulation of cellular differentiation. In early amphibian embryogenesis, FGF induces the formation of ventral mesoderm[29,30] and PtdIns hydrolysis is crucial for its development[31], which suggests a mechanism by which FGF may control mesoderm formation during amphibian embryogenesis. FGF-induced PtdIns hydrolysis might also be involved in the control of cell shape and morphology. Phosphatidylinositol(4,5)-bisphosphate is able to interact specifically with the actin-binding protein profilin[32], which may increase the amount of monomeric actin available for polymerization and so influence cell shape and motility. Hence the Y766F FGF receptor mutant could be a powerful tool for dissecting the role of PtdIns hydrolysis in cellular responses mediated by FGF. □

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Structure of an SH2 domain of the p85α subunit of phosphatidylinositol-3-OH kinase

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RECEPTOR protein-tyrosine kinases, through phosphorylation of specific tyrosine residues, generate high-affinity binding sites which direct assembly of multienzyme signalling complexes[1,2]. Many of these signalling proteins, including phospholipase Cγ, GTPase-activating protein and phosphatidylinositol-3-OH kinase, contain *src*-homology 2 (SH2) domains, which bind with high affinity and specificity to tyrosine-phosphorylated sequences[3,4]. The critical role played by SH2 domains in signalling has been highlighted by recent studies showing that mutation of specific phosphorylation sites on the platelet-derived growth factor receptor impair its association with phosphatidylinositol-3-OH kinase, preventing growth factor-induced mitogenesis[5,6]. Here we report the solution structure of an isolated SH2 domain from the 85K regulatory subunit of phosphatidylinositol-3-OH kinase, determined using multidimensional nuclear magnetic resonance spectroscopy. The structure is characterized by a central region of β-sheet flanked by two α-helices, with a highly flexible loop close to functionally important residues previously identified by site-directed mutagenesis[7,8].

Phosphatidylinositol-3-OH kinase (PtdIns-3-OH kinase) is a heterodimeric protein, consisting of a regulatory subunit of M_r 85K and a 110K catalytic subunit (p85α and p110, respectively)[9,10]. Through its SH2 domains, p85α binds to activated protein-tyrosine kinases and acts as an adapter protein, mediating the recruitment of the catalytic p110 subunit to the plasma membrane. The amino-terminal SH2 domain, included within residues 314–431 of bovine p85α, was expressed as a glutathione *S*-transferase fusion protein[11]. Compared to SH2 domains as defined by sequence homology[3,12] the 118 amino-acid fragment used in this study contains a total of ~20 amino acids in N- and carboxy-terminal extensions. These extra residues were included because a recombinant fragment corresponding to the consensus domain displayed at least a 10-fold reduced affinity for binding to a phosphorylated peptide corresponding to the sequence surrounding Tyr 751 of the platelet-derived growth factor (PDGF) receptor. The longer recombinant SH2 domain displayed enhanced binding ($K_d = 3 \times 10^{-9}$ M, data not shown) and solubility, suggesting that the N- and C-terminal extensions contribute to the correct folding and stability of the SH2 domain. The affinity-purified fusion protein was cleaved using thrombin, a procedure that resulted in the addition of two amino acids (Gly, Ser) at the N terminus of the SH2 domain-containing fragment. ^{15}N-labelled material was purified in the same way from bacteria grown in minimal medium[13] containing $^{15}NH_4Cl$

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